Knockdown of phosphatase and tensin homolog (PTEN) inhibits fatty acid oxidation and reduces very low density lipoprotein assembly and secretion in calf hepatocytes

Bichen Zhao, Chunhai Luo, Menglong Zhang, Feifei Xing, Shengbin Luo, Shixin Fu,* and Xudong Sun*

Heilongjiang Provincial Key Laboratory of Prevention and Control of Bovine Diseases, College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, No. 5 Xinyang Road, Sartu District, Daqing, Heilongjiang Province 163319, China

ABSTRACT

Dairy cows with fatty liver exhibit hepatic lipid accumulation and disturbances in fatty acid oxidation and lipid transport. Phosphatase and tensin homolog (PTEN), a lipid phosphatase, regulates intrahepatic fatty acid oxidation and lipid transport in mice. Whether PTEN play a role in fatty acid oxidation and very low density lipoprotein (VLDL) assembly in calf hepatocytes are unknown. Hepatocytes isolated from 3 healthy female Holstein calves (1 d old, 30–40 kg) were infected with empty adenovirus with green fluorescent protein for 48 h (Ad-GFP group) or infected with PTEN knockdown adenovirus for 48 h (Ad-shPTEN group), or cultured in RPMI-1640 without Ad-shPTEN or Ad-GFP (control group). Compared with the Ad-GFP group, PTEN knockdown decreased mRNA and protein abundance and the activity of fatty acid oxidation-related molecules, including acyl-coA synthetase long-chain 1, carnitine palmitoyltransferase 1, carnitine palmitoyltransferase 2, and 3-hydroxy acyl-coA dehydrogenase. Furthermore, PTEN knockdown decreased mRNA and protein abundance of VLDL assembly-related molecules, including apolipoprotein B100, apolipoprotein E, microsomal triglyceride transfer protein, and low density lipoprotein receptor. Importantly, PTEN knockdown promoted triglyceride accumulation in hepatocytes and reduced the VLDL content in culture medium. A subsequent study was conducted on the following 4 groups: cells infected with Ad-GFP for 48 h and then treated with 2% BSA for another 24 h (Ad-GFP + BSA); cells infected with Ad-GFP for 48 h and then treated with 1.2 mM free fatty acids (FFA) and 2% BSA for another 24 h (Ad-GFP + 1.2 mM FFA); cells infected with Ad-shPTEN for 48 h and then treated with 2% BSA for another 24 h (Ad-shPTEN +

BSA); cells infected with Ad-shPTEN for 48 h and then treated with 1.2 mM FFA and 2% BSA for another 24 h (Ad-shPTEN + 1.2 mM FFA). Compared with Ad-GFP + BSA, the abundances of PTEN and of fatty acid oxidation- and VLDL assembly-related proteins were lower in the Ad-GFP + 1.2 mM FFA group. Importantly, PTEN knockdown heightened the increase in triglyceride accumulation of hepatocytes and the decrease in VLDL content in culture medium induced by FFA. Overall, these in vitro data indicate that FFA inhibits PTEN expression, leading to triglyceride accumulation and the inhibition of VLDL assembly in calf hepatocytes. These findings suggest that PTEN may be a potential therapeutic target for FFA-induced hepatic steatosis in dairy cows.

Key words: phosphatase and tensin homolog (PTEN), fatty acid oxidation, very low density lipoprotein (VLDL), dairy cow

INTRODUCTION

Fatty liver is a common metabolic disease that affects up to 50% of high-producing dairy cows during the transition period (Bobe et al., 2004). During the early lactation period, due to negative energy balance, dairy cows mobilize fat depots to provide fatty acids as an energy fuel (Ntallaris et al., 2017). Excessive fat mobilization and a subsequent increase in the plasma concentration of free fatty acids (FFA) during the early postpartum period are important causes of fatty liver. Furthermore, FFA derived from the mobilization of adipose tissue are more likely to be converted to triglyceride (TG) in the liver during the early postpartum period, which increases the likelihood of fatty liver development in dairy cows (Litherland et al., 2011). Fatty liver occurs when the rate of fatty acid uptake exceeds the oxidation or disposal capacity for fatty acids. Importantly, compared with monogastric animals such as humans and mice, dairy cows show an inherently slower secretion rate of hepatic very low density lipoprotein (VLDL; Oikawa et al., 2017). Furthermore,

Received November 17, 2019.

Accepted July 8, 2020.

*Corresponding authors: fushixin@163.com and sunxudong0323@163.com

the synthesis and secretion of VLDL were significantly decreased in the livers of dairy cows with fatty liver (Kato, 2002; Bernabucci et al., 2004). Collectively, these processes result in a high natural incidence of fatty liver in dairy cows, especially in the transition period.

Phosphatase and tensin homolog (**PTEN**) is a protein tyrosine phosphatase that regulates the expression of lipid metabolism molecules in the liver, and it is associated with the development of fatty liver. The liver-specific deletion of PTEN in mice enhances fatty acids synthesis and induces fatty liver (Stiles et al., 2004). Knockdown of PTEN increases lipid substrate availability under reduced hepatic lipoprotein production capacity, which can rapidly lead to fatty liver (Qiu et al., 2008; Deng et al., 2019). Thus, we hypothesized that PTEN may play an important role in the regulation of lipid metabolism and VLDL assembly and secretion in the bovine liver.

The accumulation of TG and the disorder of fatty acid oxidation and lipid transport characterize fatty liver in dairy cows. As crucial mediators of fatty acid oxidation, hepatic acyl-coA synthetase long-chain 1 (*ACSL1*) and carnitine palmitoyltransferase 1 (*CPT1*) mRNA expression is markedly upregulated in transitional cows (Weber et al., 2013; Ha et al., 2017). Loo et al. (2007) found that the mRNA abundance of apolipoprotein B100 (*ApoB*), a VLDL assembly-related molecule, was significantly decreased in cows with fatty liver. Therefore, these studies demonstrated that alterations in fatty acid oxidation and VLDL assembly-related molecules play crucial roles in the development of fatty liver. Given that PTEN plays important roles in the regulation of intrahepatic fatty acid oxidation and lipid transport in rodents, we hypothesized that PTEN may also mediate the alterations in fatty acid oxidation and VLDL assembly induced by fatty acids in calf hepatocytes.

MATERIALS AND METHODS

Primary Calf Hepatocyte Culture

The study protocol was approved by the Ethics Committee on the Use and Care of Animals of Jilin University (Changchun, China). Intact liver tissue was collected from 3 Holstein calves (1 d old, 30–40 kg, fasting, female, and healthy). The caudate lobe of the liver was obtained through surgical excision under sterile conditions. Subsequently, the hepatocytes were isolated by the collagenase IV perfusion method as previously described (Li et al., 2014). In brief, the surface of the calf liver caudate lobe was rinsed clean with perfusate A (2.5 mM glucose, 0.5 mM EDTA, 6.7 mM KCl, 10

mM HEPES, and 140 mM NaCl; pH 7.2–7.4; 37°C) under sterile conditions. Blood vessels on the caudal surface of the liver that could be easily perfused were selected and perfused with perfusate A at a flow rate of 50 mL/min for 20 min. The perfusion stopped when the liquid became transparent. The liver was then perfused with perfusate B (2.5 mM glucose, 5 mM CaCl₂, 6.7 mM KCl, 30 mM HEPES, and 140 mM NaCl; pH 7.2–7.4; 37°C) at a flow rate of 50 mL/min until the liquid became clear. Subsequently, the liver was perfused with collagenase IV solution (0.1 g of collagenase IV dissolved in 0.5 L of perfusion solution B, pH 7.2–7.4, 37°C) for 20 min at a flow rate of 25 mL/min to dissociate the liver tissue structure. The digested liver was transferred to a sterile beaker, and 100 mL of fetal bovine serum (**FBS**; HyClone Laboratories, Logan, UT) was added to stop collagenase digestion. The liver was cut open, the undigested liver tissue was discarded, and the adipose tissue and connective tissue were removed. The remainder of the liver parenchyma was cut into pieces and filtered sequentially with 100-mesh (150-μm) and 200-mesh (75-μm) cell sieves. The hepatocyte suspension was washed twice with RPMI-1640 basic medium (HyClone Laboratories) and centrifuged at 500 × *g* for 5 min. After the cells were resuspended, the isolated hepatocytes were seeded into 6-well culture plates at a concentration of 1 × 10⁶ cells/mL using adherent medium (RPMI-1640 basic medium with 10% FBS and 10^{−3} mM dexamethasone supplemented with 10^{−3} mM insulin and 10 μg/mL vitamin C and incubated at 37°C under 5% CO₂. After 4 h, cell adherence was observed, and the medium was replaced with RPMI-1640 basic medium containing 10% FBS and 1% streptomycin. The medium was replaced with fresh medium every 24 h.

Identification of Primary Calf Hepatocytes

To identify isolated primary calf hepatocytes, we applied immunofluorescence to detect the expression of cytokeratin-18 (CK-18) a commonly used marker protein of hepatocytes (Jiang et al., 2013). After 72 h, the cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. To block nonspecific binding, hepatocytes were incubated with 3% Albumin Fraktion V (Carl Roth, Karlsruhe, Germany), 5% normal goat serum (Sigma, Darmstadt, Germany), and 0.5% Triton in PBS (PAA, Cölbe, Germany) for 30 min at room temperature. The cells were then exposed to a mouse monoclonal CK-18 antibody (ab668, 1:400; Abcam, Cambridge, UK) at 4°C in a humidified chamber overnight. The cells were then rinsed 4 times with PBS and incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG

(A0568, 1:500; Beyotime, Shanghai China) for 1 h at room temperature. After the cells were washed 3 times and covered with a glass coverslip, images were acquired with a laser confocal microscope (LSM 5 Pascal, Zeiss, Oberkochen, Germany). The results showed that the cytoplasm of more than 95% of the cells contained CK-18 (Supplemental Figure S1; <https://doi.org/10.3168/jds.2019-17920>).

Construction of Recombinant Adenoviruses and FFA Preparation

The empty adenovirus vector (negative control; adenovirus-green fluorescent protein, **Ad-GFP**) and PTEN knockdown adenovirus (**Ad-shPTEN**) were constructed by Hanbio (Shanghai, China). Briefly, to construct the PTEN expression plasmid, full-length bovine PTEN was amplified from cDNA extracted from mammary tissue using PCR and subcloned into the pcDNA3.1 (+) vector (Invitrogen, Carlsbad, CA). The primers are listed in Supplemental Table S1 (<https://doi.org/10.3168/jds.2019-17920>). The integrity of the plasmid DNA was verified by DNA sequencing. To construct the short hairpin RNA (**shRNA**) plasmid targeting PTEN, a pair of oligonucleotides specific for the bovine PTEN sequence were chemically synthesized and subcloned into the *KpnI*/*Bam*HI sites of the pMD-18T vector (TaKaRa Biotechnology Co., Ltd., Tokyo, Japan). The shRNA sequence was CACAGUUA-GAACUUAUCAATTUUGAUAAGUUCUAAACUGUGTT. Subsequently, plasmids capable of increasing or decreasing the expression of PTEN were directly subcloned into the pShuttle-Basic-EGFP vector (Invitrogen). The resulting plasmid, called pShuttle-GFP-PTEN-shRNA, was then transferred to a pAdxsi vector (Invitrogen) to generate a PTEN-silencing adenovirus (Ad-shPTEN). We then transfected this vector into human embryonic kidney 293 (HEK293) cells. A virus titer of 1.2×10^{10} pfu/mL was achieved after packaging, collection, and amplification. Ad-shPTEN was stored at -80°C for subsequent experiments.

The concentration of FFA (1.2 mM) was based on our previous studies (Gao et al., 2018; Jia et al., 2019). The composition of the stock FFA mix (52.7 mM) was designed to resemble that of FFA released from adipose tissue solution (Du et al., 2018b) and contained oleic acid (*cis*-9 18:1, 22.9 mM; Sigma-Aldrich), linoleic acid (*cis*-9,*cis*-12 18:2, 2.6 mM; Sigma-Aldrich), palmitic acid (16:0, 16.8 mM; Sigma-Aldrich), stearic acid (18:0, 7.6 mM; Sigma-Aldrich), and palmitoleic acid (*cis*-9 16:1, 2.8 mM; Sigma-Aldrich). The FFA stock solution was prepared by diluting fatty acids in 0.1 M KOH at 60°C , and then the pH of the solution was adjusted to 7.4 with 1 M HCl.

Adenovirus Transfections and FFA Treatment

After 72 h, the hepatocytes were starved in serum-free RPMI-1640 basic medium for 12 h. For adenovirus transfections experiments, hepatocytes were divided into the 3 groups: empty adenovirus (Ad-GFP) group (cells infected with Ad-GFP for 48 h), PTEN knockdown adenovirus (Ad-shPTEN) group (cells infected with Ad-shPTEN for 48 h), and control group (cells cultured in RPMI-1640 without Ad-shPTEN or Ad-GFP). We used a fluorescence confocal microscope and flow cytometer to determine the optimal transfection concentration.

For adenovirus transfection and FFA treatment experiments, hepatocytes were divided into the 4 groups: (1) cells infected with Ad-GFP for 48 h and then treated with 2% BSA for another 24 h (Ad-GFP + BSA); (2) cells infected with Ad-GFP for 48 h and then treated with 1.2 mM FFA and 2% BSA for another 24 h (Ad-GFP + 1.2 mM FFA); (3) cells infected with Ad-shPTEN for 48 h and then treated with 2% BSA for another 24 h (Ad-shPTEN + BSA); (4) cells infected with Ad-shPTEN for 48 h and then treated with 1.2 mM FFA and 2% BSA for another 24 h (Ad-shPTEN + 1.2 mM FFA).

Quantitative Real-Time PCR Assay

Quantitative real-time (qRT)-PCR assays were performed as described previously (Sun et al., 2019). Total RNA was extracted from hepatocytes using TRIzol (Takara Biotechnology Co. Ltd., Dalian, China) according to the manufacturer's instructions. The RNA concentration and quality were analyzed using a K5500 MicroSpectrophotometer (Beijing Kaiao Technology Development Ltd., Beijing, China) and electrophoresis (1% agarose gels). Then, 3 μg of total RNA was reverse-transcribed into cDNA using a reverse transcription kit (TaKaRa Biotechnology Co. Ltd., Tokyo, Japan) according to the manufacturer's protocol. In our study, the average RNA integrity value was 8. The mRNA abundance was measured with the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) using a SYBR Green Plus Reagent Kit (TaKaRa Biotechnology Co. Ltd.). The conditions were as follows: initial denaturation at 94°C for 2 min, 30 cycles of amplification (denaturation at 94°C for 30 s, annealing at 60°C for 15 s, and extension at 72°C for 30 s), and extension at 72°C for 10 min. The stability of 3 housekeeping genes, *UBB*, *GAPDH*, and *ACTB*, was analyzed by BestKeeper (Pfaffl et al., 2004). The results showed that the standard deviation (SD) values of ubiquitin, *GAPDH*, and *ACTB* were all <1 , indicating stability and supporting their use as internal

control genes (Supplemental Table S2; <https://doi.org/10.3168/jds.2019-17920>). Thus, the relative expression of each target gene was normalized to that of ubiquitin, *GAPDH*, and *ACTB*. The abundance of the target genes was calculated by the $2^{-\Delta\Delta C_t}$ method. The qRT-PCR was performed 15 times for each group, and primers for each gene are shown in Supplemental Table S1.

Protein Extraction and Western Blot Analysis

Western blotting was performed as described previously (Xu et al., 2019). Total cellular protein was extracted from hepatocytes using a commercial protein extraction kit (BestBio Ltd., Shanghai, China) according to the manufacturer's instructions. Protein content was determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime, China). Proteins (30 μ g/lane) were separated by SDS-PAGE and electrotransferred onto polyvinylidene fluoride (PVDF) membranes. Next, the membranes were blocked in 3% BSA for 2 h. The blocked membranes were incubated overnight at 4°C with primary antibodies against ACTB (internal control; ab8226, 1:2,500; Abcam, Cambridge, UK), PTEN (sc-7974, 1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA), ACSL1 (ab177985, 1:2,000; Abcam), CPT1 (ab83862, 1:1,000; Abcam), carnitine palmitoyltransferase 2 (CPT2, ab103907, 1:1,000; Abcam), 3-hydroxy acyl-coA dehydrogenase (**HADH**, ab81492, 1:1,000; Abcam), ApoB100 (ab7616, 1:1,000; Abcam), apolipoprotein E (**ApoE**, sc-13521, 1:500; Santa Cruz Biotechnology), microsomal triglyceride transfer protein (**MTP**, sc-135994, 1:500; Santa Cruz Biotechnology), and low density lipoprotein receptor (**LDLR**, sc-18823, 1:500; Santa Cruz Biotechnology). After washing 4 times, the blocked membranes were incubated in a horseradish peroxidase-conjugated anti-goat, anti-mouse, or anti-rabbit antibody (Boster, Wuhan, China) at room temperature for 45 min. Immunoreactive bands were detected using an enhanced chemiluminescence solution (Pierce Biotechnology Inc., Chicago, IL). Finally, the bands were visualized using the ChemiDoc MP System (Bio-Rad Laboratories, Hercules, CA).

Determination of Enzyme Activity

The activities of ACSL1, CPT1, and CPT2 (ml063470, ml076616, ml063474, Shanghai Enzyme-linked Biotechnology Co. Ltd., Shanghai, China) and HADH (GMS50068.2, Shanghai Genmed Co. Ltd., Shanghai, China) in hepatocytes were measured by using enzymatic colorimetric method kits according to the manufacturer's protocols. In brief, the hepatocytes were washed with PBS 3 times, harvested, lysed with

lysis buffer (Sangon Biotech Co. Ltd., Shanghai, China) in an ice bath and shaken every 5 min. The lysates were centrifuged at $14,000 \times g$ for 10 min at 4°C. When ACSL1, CPT1, CPT2, and HADH combined with their respective substrates and catalyzed their respective reactions, changes in absorbance were observed. The substrate of ACSL1, CPT1, and CPT2 is palmitoyl-CoA; the substrate of HADH is 3-ketoacyl-CoA.

Determination of TG Content

After washing twice in ice-cold PBS, the hepatocytes were harvested using a cell scraper and transferred to a 1.5-mL centrifuge tube. Subsequently, the cells were lysed with lysis buffer (Sangon Biotech Co. Ltd.) in an ice bath and shaken every 5 min. Then, the concentration of TG in the hepatocytes was measured using a TG detection kit (A110-2-1, Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) according to the manufacturer's protocol. The total protein concentration in the hepatocytes was analyzed using the BCA protein assay kit (Beyotime Co. Ltd.).

Determination of VLDL Content

Cell culture supernatant was collected from the 6-well plates and supernatants were centrifuged at $1,000 \times g$ for 15 min to remove debris. The content of VLDL in the supernatants was measured using an ELISA kit (E11V0006, Shanghai Bluegene Biotech Co. Ltd., Shanghai, China) according to the manufacturer's protocol.

Statistical Analysis

All experiments were performed in triplicate, and the results are reported as means $\pm$ standard error of the means. The data were analyzed using SPSS 22.0 (IBM Corp., Armonk, NY). Statistical significance was evaluated by one-way ANOVA with Duncan's test for post hoc analysis. $P < 0.05$ and $P < 0.01$ were considered significant and highly significant, respectively.

RESULTS

Adenovirus-Mediated Knockdown of PTEN in Calf Hepatocytes

The Ad-GFP vector was transfected into calf hepatocytes (Figure 1A and B) and, after 48 h, the positive rate of expression of Ad-GFP was analyzed by flow cytometry. When the multiplicity of infection (MOI) exceeded 100, the positive rate of expression of Ad-GFP became stable (Figure 1C). The mRNA and pro-

tein expression of PTEN in the Ad-shPTEN group was significantly lower than that in the control and negative control groups ($P < 0.01$; Figure 1D–F).

Effect of PTEN Knockdown on Expression of Fatty Acid Oxidation-Related Molecules

Knockdown of PTEN sharply decreased the mRNA abundance of *ACSL1* ($P < 0.01$), *CPT1* ($P < 0.05$), *CPT2* ($P < 0.01$), and *HADH* ($P < 0.05$; Figure 2). Similarly, the protein abundance of *ACSL1* ($P < 0.01$), *CPT1* ($P < 0.01$), *CPT2* ($P < 0.01$), and *HADH* ($P < 0.05$) was significantly decreased in the Ad-shPTEN group compared with the Ad-GFP group (Figure 3). Furthermore, PTEN knockdown markedly decreased the enzyme activity of *ACSL1* ($P < 0.01$), *CPT1* ($P < 0.05$), *CPT2* ($P < 0.05$), and *HADH* ($P < 0.05$; Figure 4). These results suggest that the knockdown of PTEN in calf hepatocytes inhibits fatty acid oxidation.

Effect of PTEN Knockdown on Expression of VLDL Assembly- and Secretion-Related Molecules

The mRNA abundances of *ApoB* ($P < 0.01$), *ApoE* ($P < 0.01$), *MTP* ($P < 0.01$), and *LDLR* ($P < 0.05$)

were significantly lower in the Ad-shPTEN group than in the Ad-GFP group (Figure 5). In addition, treatment with Ad-shPTEN sharply decreased the protein abundance of *ApoB100* ($P < 0.05$), *ApoE* ($P < 0.05$), *MTP* ($P < 0.05$), and *LDLR* ($P < 0.05$; Figure 6). Overall, these results suggest that PTEN knockdown in calf hepatocytes inhibits the assembly and secretion of VLDL.

Effect of PTEN Knockdown on Contents of TG and VLDL

To confirm the effects of PTEN knockdown on fatty acid metabolism in calf hepatocytes, the TG content in hepatocytes and the VLDL content in culture supernatant were measured. The results revealed that TG content in hepatocytes was significantly upregulated in the Ad-shPTEN group compared with the control and negative control groups ($P < 0.05$; Figure 7A). In contrast, PTEN knockdown sharply decreased the level of VLDL in the supernatant ($P < 0.05$; Figure 7B). These results clearly indicate that PTEN knockdown inhibits VLDL secretion and induces TG accumulation in calf hepatocytes.

Figure 1. Adenovirus-mediated knockdown of phosphatase and tensin homolog (PTEN) in calf hepatocytes. (A) Fluorescence of calf hepatocytes in the control group (uninfected). (B) Fluorescence of calf hepatocytes in the Ad-shPTEN group (infected with PTEN knockdown adenovirus Ad-shPTEN). (C) Positive rate of cells in different multiplicities of infection (MOI). (D–F) mRNA and protein expression of PTEN. Groups were compared using one-way ANOVA with a Duncan correction. The data are presented as mean $\pm$ SEM; ** $P < 0.01$; difference between groups.

Effect of FFA and PTEN Knockdown on Expression of Fatty Acid Oxidation- and VLDL Assembly-Related Molecules

The protein abundance of PTEN was significantly lower in the Ad-GFP + 1.2 mM FFA group than in the Ad-GFP + BSA group ($P < 0.05$; Figure 8A). Furthermore, treatment with 1.2 mM FFA (Ad-GFP + 1.2 mM FFA group) significantly decreased the protein abundance of fatty acid oxidation-related molecules, including CPT2 and HADH ($P < 0.01$, Figure 8A), and VLDL assembly-related molecules, including ApoB, ApoE, MTP, and LDLR ($P < 0.01$; Figure 8C). The TG content in hepatocytes was greater ($P < 0.01$) in the Ad-GFP + 1.2 mM FFA cultures than in the Ad-GFP + BSA cultures, whereas the VLDL content in the supernatant was lower ($P < 0.01$) (Figure 8E and F).

The protein abundance of PTEN was markedly decreased in the Ad-shPTEN + 1.2 mM FFA group compared with the Ad-GFP + 1.2 mM FFA group ($P < 0.01$, Figure 8A). Moreover, compared with the Ad-

GFP + 1.2 mM FFA group, the combination of PTEN knockdown and 1.2 mM FFA treatment decreased the protein abundance of fatty acid oxidation-related molecules, including ACSL1 ($P < 0.01$), CPT1 ($P < 0.01$), CPT2 ($P < 0.05$), and HADH ($P < 0.01$), and VLDL assembly-related molecules, including ApoB ($P < 0.01$), MTP ($P < 0.01$), and LDLR ($P < 0.01$, Figure 8A and C). In addition, compared with the Ad-GFP + 1.2 mM FFA group, treatment with Ad-shPTEN and FFA sharply increased the content of TG in hepatocytes and decreased the content of VLDL in the supernatant ($P < 0.01$; Figure 8E and F). These results suggest that PTEN knockdown exacerbates the disorder of fatty acid oxidation and VLDL assembly in the presence of FFA.

DISCUSSION

Fatty liver is usually associated with negative energy balance-induced lipid metabolism disorder. Accumulation of TG in hepatocytes results primarily from an

Figure 2. Effect of phosphatase and tensin homolog (PTEN) knockdown on relative mRNA expression of fatty acid oxidation-related genes. The Ad-shPTEN group was infected for 48 h with an adenovirus that downregulated PTEN (Ad-shPTEN), the control group was cultured in RPMI-1640 basic medium without the adenovirus or with empty adenovirus vector, and the Ad-GFP group was infected with an empty adenovirus vector plus green fluorescent protein (GFP) for 48 h. The mRNA expression of (A) acyl-CoA synthetase long-chain 1 (*ACSL1*), (B) carnitine palmitoyltransferase 1 (*CPT1*), (C) carnitine palmitoyltransferase 2 (*CPT2*), and (D) 3-hydroxy acyl-CoA dehydrogenase (*HADH*) was measured by quantitative real-time (qRT)-PCR. Groups were compared using one-way ANOVA with a Duncan correction. The data are presented as mean $\pm$ SEM; * $P < 0.05$, ** $P < 0.01$: difference between groups.

Figure 3. Effect of phosphatase and tensin homolog (PTEN) knockdown on expression of fatty acid oxidation-related proteins. The Ad-shPTEN group was infected for 48 h with an adenovirus that downregulated PTEN (Ad-shPTEN), the control group was cultured in RPMI-1640 basic medium without the adenovirus or empty adenovirus vector, and the Ad-GFP group was infected with an empty adenovirus vector plus green fluorescent protein (GFP) for 48 h. (A) Western blot analysis of acyl-CoA synthetase long-chain 1 (ACSL1), carnitine palmitoyltransferase 1 (CPT1), carnitine palmitoyltransferase 2 (CPT2), and 3-hydroxy acyl-CoA dehydrogenase (HADH). Relative protein expression of (B) ACSL1, (C) CPT1, (D) CPT2, and (E) HADH. Groups were compared using one-way ANOVA with a Duncan correction. The data are presented as mean $\pm$ SEM; * $P < 0.05$, ** $P < 0.01$: difference between groups.

imbalance among fatty acid oxidation, TG synthesis, and TG export via VLDL (McCarthy et al., 2010). Moreover, PTEN plays important roles in the development of hepatic TG accumulation in humans and mice (Kachaylo et al., 2017; Zhao et al., 2019). In rodents, CRISPR/Cas9-based PTEN knockout was shown to induce hepatic lipid accumulation (Gao and Liu, 2017). As in nonruminants, the higher TG content and the lower abundance and activity of fatty acid oxidation- and VLDL assembly-related molecules in the PTEN knockdown cultures compared with control cultures highlighted the negative effect of PTEN on TG accumulation in calf hepatocytes. Notably, excessive fatty acid can inhibit VLDL assembly and lead to the disorder of fatty acid oxidation, thereby inducing TG accumulation in the liver of dairy cows (Du et al., 2018a). In agreement with *in vivo* data, FFA treatment increased the TG content in hepatocytes and reduced VLDL assembly. Because PTEN knockdown heightened the increase in TG content induced by FFA and altered the abundance of fatty acid oxidation- and

VLDL assembly-related molecules, the availability of FFA within calf hepatocytes might be a signal that regulates PTEN.

β -Oxidation is the lipid catabolism process by which fatty acids are broken down in mitochondria to generate energy. However, incomplete β -oxidation leads to the re-esterification of fatty acids into TG, which is one of the important causes of TG accumulation in the liver. Yamamoto-Kataoka et al. (2015) reported that the liver-specific deletion of PTEN in mice enhanced TG accumulation in the liver. Interestingly, we found that PTEN knockdown significantly increased the TG content in calf hepatocytes. Our data, together with the findings of Yamamoto-Kataoka et al. (2015), indicate that decreased PTEN induces TG accumulation in hepatocytes, which may contribute to the development of fatty liver in dairy cows. Although the abundance of PTEN in the fatty livers of dairy cows was not measured, our data suggest that a decrease in the expression of PTEN may contribute to fatty liver development during FFA stimulation.

Figure 4. Effect of phosphatase and tensin homolog (PTEN) knockdown on the activity of fatty acid oxidation-related enzymes. The Ad-shPTEN group was infected for 48 h with an adenovirus that downregulated PTEN (Ad-shPTEN), the control group was cultured in RPMI-1640 basic medium without the adenovirus or the empty adenovirus vector, and the Ad-GFP group was infected with an empty adenovirus vector plus green fluorescent protein (GFP) for 48 h. Activity of (A) acyl-CoA synthetase long-chain 1 (ACSL1), (B) carnitine palmitoyltransferase 1 (CPT1), (C) carnitine palmitoyltransferase 2 (CPT2), and (D) 3-hydroxy acyl-CoA dehydrogenase (HADH). Groups were compared using one-way ANOVA with a Duncan correction. The data are presented as mean $\pm$ SEM; * P < 0.05, ** P < 0.01; difference between groups.

The process of β -oxidation is regulated by various enzymes, including ACSL1, CPT1, and CPT2 (Zhou et al., 2015; Guo et al., 2018). Located in the outer mitochondrial membrane, ACSL1 is the rate-limiting enzyme for fatty acid activation (Bonnefont et al., 2004; Warfel et al., 2017); it catalyzes long-chain fatty acids to long-chain acyl-coA; then, fatty acyl-coA is transported to mitochondria by CPT1 and CPT2 for β -oxidation (Eaton, 2002). Work in nonruminants has demonstrated that a lack of hepatic PTEN decreased the activities of CPT1 and CPT2 (Piguet et al., 2009). In the present study, the lower abundance and enzyme activity of ACSL1, CPT1, and CPT2 in PTEN knockdown cultures underscored that PTEN knockdown decreased the β -oxidation of fatty acids and in turn led to TG accumulation in hepatocytes. In fact, studies on cows with fatty liver have suggested that low hepatic CPT1 protein abundance is linked to impeded hepatic fatty acid oxidation (Loor et al. (2005). In addition, HADH is an intramitochondrial enzyme that catalyzes the penultimate step of β -oxidation (Schulz et al., 2011).

In the present study, PTEN knockdown decreased the abundance and enzyme activity of HADH in calf hepatocytes; this finding, together with the observed decrease in mRNA abundance of *HADH* in the liver of cows with negative energy balance (McCarthy et al., 2010), indicated that PTEN contributes to hepatic TG accumulation. Collectively, the decrease in the expression of fatty acid oxidation molecules induced by PTEN knockdown in calf hepatocytes supports the argument that PTEN participates in the regulation of TG accumulation by regulating fatty acid oxidation.

Export of TG via VLDL is an important lipid disposal pathway in the liver. Compared with other species, dairy cows have a very slow rate of hepatic VLDL secretion, which makes them more susceptible to fatty liver (Hocquette and Bauchart, 1999). Herdt and Smith (1996) reported that the plasma concentration of VLDL in dairy cows with fatty liver was significantly lower than that in healthy cows. The decrease in VLDL content in the supernatant of cultures with low PTEN expression suggests that PTEN may also contribute to

Figure 5. Effect of phosphatase and tensin homolog (PTEN) knockdown on mRNA expression of very low density lipoprotein (VLDL) assembly and secretion-related genes. The Ad-shPTEN group was infected for 48 h with an adenovirus that downregulated PTEN (Ad-shPTEN), the control group was cultured in RPMI-1640 basic medium without the adenovirus or the empty adenovirus vector, and the Ad-GFP group was infected with an empty adenovirus vector plus green fluorescent protein (GFP) for 48 h. The mRNA expression of (A) apolipoprotein B100 (*ApoB100*), (B) apolipoprotein E (*ApoE*), (C) microsomal triglyceride transfer protein (*MTP*), and (D) low density lipoprotein receptor (*LDLR*) was measured by quantitative real-time PCR. Groups were compared using one-way ANOVA with a Duncan correction. The data are presented as mean $\pm$ SEM; * P < 0.05, ** P < 0.01: difference between groups.

Figure 6. Effect of phosphatase and tensin homolog (PTEN) knockdown on relative expression of very low density lipoprotein (VLDL) assembly and secretion-related proteins. The Ad-shPTEN group was infected for 48 h with an adenovirus that downregulated PTEN (Ad-shPTEN), the control group was cultured in RPMI-1640 basic medium without the adenovirus or the empty adenovirus vector, and the Ad-GFP group was infected with an empty adenovirus vector plus green fluorescent protein (GFP) for 48 h. (A) Western blot analysis of apolipoprotein B100 (ApoB), apolipoprotein E (ApoE), microsomal triglyceride transfer protein (MTP), and low density lipoprotein receptor (LDLR). Protein expression of (B) ApoB, (C) ApoE, (D) MTP, and (E) LDLR. Groups were compared using one-way ANOVA with a Duncan correction. The data are presented as mean $\pm$ SEM; * P < 0.05, ** P < 0.01: difference between groups.

the accumulation of TG by regulating VLDL synthesis and assembly.

Apolipoprotein B100 and ApoE are the most important structural components of VLDL (Bobe et al., 2004) and play crucial roles in the accumulation of TG (Curtiss and Boisvert, 2000). Schlegel et al. (2012) demonstrated that the liver TG content of postpartum dairy cows was negatively correlated with the ApoB100 and ApoE content in blood. Qiu et al. (2008) reported that the hepatic expression of ApoB and ApoE was significantly downregulated in liver-specific PTEN knockout mice, suggesting the role of PTEN in the regulation of the abundance of ApoB and ApoE. Consistent with previous findings in mouse livers (Qiu et al., 2008), we found in the present study that PTEN knockdown decreased the abundance of ApoB and ApoE in calf hepatocytes, which in turn decreased VLDL synthesis and assembly and induced TC accumulation. Notably, a decrease in the hepatic mRNA abundance of ApoB in dairy cows with fatty liver has been reported (Marcos et al., 1990), suggesting that PTEN may participate in the development of fatty liver by affecting the structural stability of VLDL.

Microsomal triglyceride transfer protein and LDLR are important regulatory proteins of the synthesis of VLDL: MTP is essential for the synthesis and secretion of VLDL (Sundaram and Yao, 2010), promoting the assembly of ApoB and lipoproteins, whereas LDLR inhibits the secretion of ApoB and internalizes apoE- and apoB-containing lipoproteins, including LDL and VLDL (Go and Mani, 2012). The alterations in the abundances of MTP and LDLR contributed to the hepatic TG accumulation in cows. Moreover, lower hepatic mRNA expression of *MTP* and *LDLR* was observed in cows with fatty liver than in healthy cows

(Bremmer et al., 2000; Loor et al., 2007). In addition to the lower VLDL content, the lower abundance of MTP and LDLR in PTEN knockdown cultures than in control cultures suggested that PTEN knockdown inhibited VLDL assembly and secretion, thereby increasing TG accumulation in calf hepatocytes.

Fatty liver is associated with excessive fatty acids in the blood (Li et al., 2015). In rodents, a high concentration of FFA causes steatosis and alters the abundance of molecules involved in hepatic lipid metabolism (Jump et al., 2005). These findings were confirmed in vitro by the downregulation of fatty acid oxidation-related molecules (ACSL1, CPT2, and HADH) and VLDL assembly-related molecules (ApoB, ApoE, MTP, and LDLR) in calf hepatocytes incubated with FFA. Consistent with our findings, several studies have reported that the protein abundances of ACSL1, CPT2, ApoB, ApoE, and MTP in calf hepatocytes were reduced by FFA stimulation (Li et al., 2013; Jia et al., 2019). Thus, as in nonruminants, high content of fatty acids disrupts lipid homeostasis in the livers of dairy cows, and it is notable that PTEN also mediates this biological process. The fact that the downregulation of PTEN and related molecules induced by fatty acid challenge was significantly aggravated by PTEN knockdown led us to suggest that PTEN partly mediates the development of fatty acid-induced TG accumulation and the disruption of VLDL assembly.

The acknowledged limitation of this study is that the calf hepatocyte model used in the present study might not be representative of adult bovine hepatocytes: there may be several differences between adult bovine hepatocytes and calf hepatocytes, including cell viability, cell function, and hormone sensitivity. Another limitation of the present study is that because of the lack of

Figure 7. Effect of phosphatase and tensin homolog (PTEN) knockdown on triglyceride (TG) and very low density lipoprotein (VLDL) contents. The Ad-shPTEN group was infected for 48 h with an adenovirus that downregulated PTEN (Ad-shPTEN), the control group was cultured in RPMI-1640 basic medium without the adenovirus or the empty adenovirus vector, and the Ad-GFP group was infected with an empty adenovirus vector plus green fluorescent protein (GFP) for 48 h. (A) Content of TG in calf hepatocytes; (B) content of VLDL in the supernatant. Groups were compared using one-way ANOVA with a Duncan correction. The data are presented as mean $\pm$ SEM; * P < 0.05: difference between groups.

Figure 8. Effect of free fatty acids (FFA) and phosphatase and tensin homolog (PTEN) knockdown on the expression of fatty acid oxidation and very low density lipoprotein (VLDL) assembly genes. The cells were divided into 4 groups: (1) cells infected with Ad-GFP (adenovirus vector with green fluorescent protein) for 48 h and then treated with 2% BSA for another 24 h (Ad-GFP + BSA); (2) cells infected with Ad-GFP for 48 h and then treated with 1.2 mM FFA and 2% BSA for another 24 h (Ad-GFP + 1.2 mM FFA); (3) cells infected with Ad-shPTEN (adenovirus that downregulated PTEN) for 48 h and then treated with 2% BSA for another 24 h (Ad-shPTEN + BSA); (4) cells infected with Ad-shPTEN for 48 h and then treated with 1.2 mM FFA and 2% BSA for another 24 h (Ad-shPTEN + 1.2 mM FFA). (A) Western blot analysis of PTEN, acyl-CoA synthetase long-chain 1 (ACSL1), carnitine palmitoyltransferase 1 (CPT1), carnitine palmitoyltransferase 2 (CPT2), and 3-hydroxy acyl-CoA dehydrogenase (HADH). (B) Relative protein abundance of PTEN, ACSL1, CPT1, CPT2, and HADH. (C) Western blot analysis of apolipoprotein B100 (ApoB100), apolipoprotein E (ApoE), microsomal triglyceride transfer protein (MTP), and low density lipoprotein receptor (LDLR). (D) Relative protein abundance of ApoB100, ApoE, MTP, and LDLR. (E) Content of triglyceride (TG) in calf hepatocytes. (F) Content of VLDL in the supernatant. The data of the Ad-GFP group were used to normalize the data of each treatment group. Groups were compared using one-way ANOVA with a Duncan correction. The data are presented as mean $\pm$ SEM; * P < 0.05, ** P < 0.01: difference between groups.

separation by density gradient or other methods, we cannot ensure that nonparenchymal cells are not present. Adult bovine hepatocytes are likely subjected to challenge with long-term and dynamic concentrations of FFA during early lactation. In the present study, calf hepatocytes were stimulated by a single concentration of FFA for a defined period, so whether the calf hepatocyte model used in this study mirrors in vivo conditions experienced by periparturient cows with fatty liver remains unknown. Therefore, further studies in dairy cows with fatty liver and in adult bovine hepatocytes should be performed to better address the relevance of the findings in the calf hepatocytes model from the present study.

CONCLUSIONS

Free fatty acids decreased PTEN abundance, which inhibited the oxidative metabolism of fatty acids and the assembly and secretion of VLDL, thereby inducing TG accumulation in calf hepatocytes. Accordingly, PTEN is a potential therapeutic target for the disorder of lipid accumulation and lipid metabolism induced by fatty acids in calf hepatocytes.

ACKNOWLEDGMENTS

This work was supported by the Key Scientific Research Program of the General Bureau of State Farms of Heilongjiang Province (grant no. HKKY190302), China Postdoctoral Science Foundation (Beijing, China; grant no. 2019M661316), the National Natural Science Foundation of China (Beijing, China; grant no. 31672622), the Heilongjiang Postdoctoral Science Foundation (Heilongjiang, China; grant no. LBH-Z19090), and the Personnel Foundation in Heilongjiang Bayi Agricultural University (Daqing, China; grant no. XYB201909). BZ, SF, and XS conceived the study; BZ, CL, MZ, FX, SL, SF, and XS carried out experiments and data analysis; BZ, SF, and XS interpreted the data, and BZ, SF, and XS wrote the manuscript. All authors approved the final version. The authors have stated no conflicts of interest.

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ORCIDS

Shixin Fu <https://orcid.org/0000-0001-5023-409X>